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Technologies for Estimating Viable Spores in an Aerosol Particle

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By

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ABSTRACT

There are practical and valid reasons to present biological field trial reference results as agent containing particles per liter of air (ACPLA). One fundamental characteristic of a biological threat aerosol is that the agent particles must be linked to infectivity. To be labeled as infectious, each individual particle must grow and replicate either in a host or in artificial medium. The slit sampler approach, using nutrient growth medium for particle number measurement is still the best method to approximate this relationship. However, it must be pointed out that each colony that forms on growth medium can arise from a single viable organism or an aggregate consisting of two or more individuals. It has been written that in considering threat biological aerosols, the aggregates are more relevant with respect to respirable particle studies (Day, 1981). Other biological samplers like the glass impinger (Terzieva et al. 1996) measure individual viable organisms in air. Without knowledge of the number of viable organisms per particle, comparing results from these samplers is difficult. If this number were several orders of magnitude greater than unity, misconceptions may arise in interpreting ACPLA figures, especially in threat assessment scenarios.

By using the existing DRES 90 m^3 chamber, it has been shown that low BG aerosol concentrations (5 ACPLA) can be generated reproducibly. This will be a useful feature for biological detector testing. Particle collection characteristics of the slit sampler have been studied. This is the modified version used in international refereed field trails. Virtually all trials of importance rely on results based on this collector. A statistical method was used to demonstrate sampling predictability at 95% confidence level. By using glass fiber filters mounted in a dichotomous sampler, sampling efficiencies were estimated for a variety of common aerosol collectors. This led to the selection of glass fiber filter as the optimal reference collector medium for comparative efficiency studies. The accumulated data permitted the estimation of the number of viable spores per particle. For a 2.5 to 4 μm particle, arguments have been presented for considering 4.5 as the most probable value. This is a useful conversion factor to assist comprehension of ACPLA data.

One caveat is that this value relates only to the Hudson nebulizer. The intent has been that once the proposed method is established and found acceptable for such studies, similar work can be done to characterize other generators and indeed other biological material.

Conventional aerosol facilities utilise continuous particle generators that produce a point source challenge to detectors or collectors (Keith et al. 1991). With this approach, aerosol delivery at the receiving end is an inherently random process and these authors have acknowledged the problem by attempting to solve it with distribution grids. We have described an alternative approach; by filling a chamber with a constant concentration of particulate material, replicate sampling is now possible, a feature that has been missing from other designs. The results obtained from multiple sampling permit statistical analysis to be performed. Reliable analytical results can lead to interesting predictions, for example, estimation of viable spore numbers in a particle.

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INTRODUCTION

At international field trials where biological aerosol detectors were tested, the reference data were derived from slit samplers, expressed as agent containing particles per liter of air, commonly abbreviated as "ACPLA" (Andersen and Mohr, 1996). We have shown that time series ACPLA data could be correlated satisfactorily with fluorescent data generated by an optical detector (Ho et al. 1999). From these observations, we suggested that the detector technology was essentially measuring live or culturable (Appendix 1) biological particles in air.

This optical detector, also called the Fluorescent Aerodynamic Particle Sizer or FLAPS, was designed to measure fluorescence emission signals from a single spore (Hairston et al. 1997). In practice, along with single organisms, biological aerosols usually consist of particles made up of aggregates containing two or more individuals as seen under scanning electron microscopy (Ho, 1994). Previous studies with liquid suspensions of *Bacillus subtilis* (BG) spores revealed that not every individual entity, as defined under light microscopy, is viable (Ho and Fisher. 1993). By inference, it can be assumed that a spray of this preparation would contain a mixture of viable and nonviable particles. Larger particles greater than 1 μm (native spore size) may contain numerous spores in an aggregate where only a fraction of these may be viable.

There are practical and valid reasons to present biological field trial reference results in ACPLA units. One fundamental characteristic of a biological aerosol threat is that the agent particles are linked to infectivity. To be labeled as infectious, each individual particle must grow and replicate either in a host or in artificial medium. The slit sampler approach, using nutrient growth medium for particle number measurement is still the best method to approximate this relationship. However, it must be pointed out that each colony that forms on growth medium can arise from a single viable organism or an aggregate consisting of two or more individuals. It has been written that in considering threat biological aerosols, the aggregates are more relevant in respirable particle studies (Day, 1981). Other biological samplers like the glass impinger (Terzieva et al. 1996) measure individual viable organisms in air. Without knowledge of the number of viable organisms per particle, comparing results from these samplers is difficult. If this number were several orders of magnitude greater than unity, misconceptions may arise in interpreting ACPLA figures, especially in threat assessment scenarios.

Determining the number of viable organisms in a particle requires compromises. It has been shown that various types of aerosol generators produce particles of markedly different mass median diameters (Ho, 1991). Microorganisms like Bacillus species (Elisashvili, 1984) and Erwinia species (Blake et al. 1982) are known to produce extracellular polysaccharides, also called levans, that serve as binding agents. In aerosol generation, levans may affect particle size formation although this has not been thoroughly demonstrated (Ho, 1994). Careful control of experimental conditions and reproducibility of the results is critical for proper interpretation. In this paper, we propose a method by which the average number of viable spores in a particle can be estimated.

METHODS AND MATERIAL

Aerosol chamber

Biological aerosol dissemination (spores of *Bacillus subtilis* var *niger*, BG) was accomplished with a Hudson nebulizer (model 1700, Hudson Oxygen Therapy Sales Co.,Wadsworth, OH) at 172-206 KiloPascal (25-30 psi). A suspension containing BG in the range of 5 to 30 μg plus 4 mg silica gel (Syloid 245, Davison Chemical, Baltimore, MD) per ml was used as the starting material. For each experiment, the dry BG powder was weighed out from a stock batch. For example, to prepare a 10 μg/ml suspension, 0.1 gm dry powder was weighed out (model AE 200-S, Mettler Instruments, Zurich, Switzerland) and added to 10 ml distilled water. Serial 1:10 dilutions were repeated 3 times to obtain a final concentration of 10 μg/ml. A working volume of 25 ml was used to fill the aerosol generator. Actual viable counts in the suspension were determined to obtain the source concentration.

A steady aerosol concentration in a 90.5 m^3 chamber was achieved via a feed back control loop mechanism as previously described (Ho, 1989). In this scheme, a particle sizer was used to measure aerosol concentration at every 5 seconds. The result was compared to a previously defined threshold; custom software performed the control function to regulate switching of the aerosol generator. The monitored particle size range was 0.7-1.0 μm at 1 particle per cc concentration threshold. Steady state aerosol concentration was achieved in about 15 minutes from startup. Syloid was used to provide sufficient particle counts in the aerosol to maintain efficient operation of this feedback loop. For μg/ml quantities of BG there was insufficient particles to perform this task.

Sampler collection

Critical to understanding the characteristics of the test aerosol, culturable particles in air must be captured and grown to determine the presence of "live" content. To properly characterize the BG particulate material, a number of standard biological aerosol collectors were used (Chatigny et al. 1989). Biological aerosol particles were impacted onto 15 cm nutrient agar plate situated in a slit sampler (model STA 203, New Brunswick Scientific, Edison, NJ 08818-4005). The air flow rate was 50 liter/min. Each sampler was electronically modified to rotate at 1 revolution/min. A sampler array consisted of 10 devices serially connected to function as a continuous 10 minute collector. After overnight incubation, live particles were counted as bacterial colonies by means of a flat bed scanner driven by custom software developed jointly by DRES, Dugway Proving Ground (DPG) and Spiral Biotech (model "STAR" version 1.5, Spiral Biotech, Bethesda, MD). Slit sampler data were expressed as agent containing particles per liter of air (ACPLA).

A dichotomous sampler, DS, (model 245, Andersen Samplers Inc., Atlanta, GA) operating at 17 L/min, collected particles on a set of 2 glass fiber filters representing fine and coarse fractions (Ho, 1991). After a sampling time of 20 minutes in triplicate, the were filters disrupted in 20 ml water using a wrist action shaker (model 75, Burrel corp. Pittsburgh, PA). Wire gauze disks were used to clarify the supernatant before viable spore assessment.

Standard all glass impingers (AGI30) running at 12.5 L/min were used to provide a baseline comparison (Terzieva et al. 1996). In addition, SKC glass impingers were also used (12.5 L/min model Biosampler 225-9595, SKC Inc.,

Eighty four, PA) to provide a comparison of a "new" design that has reported improved aerosol collection efficiency. Glass impinger samples were run at 5 minute integrated collection times. Routine replicate samples were obtained serially over a period about an hour. Replicates for AGI were 11 while those for SKC were 9.

Viable spore assay

Viable organisms were enumerated by the spiral plating technique as described by Hedges et al. (1978). Liquid samples were applied to standard nutrient agar plates with a spiral platter (model 4000, Spiral Biotech Inc. Bethesda, MD, http://www.spiralbiotech.com/ap4000.html). The plates were incubated overnight at 37° C and analyzed with the CASBA 4 System that combines a high-resolution line scanner and specialized Windows® based software to count microbial colonies on agar plates. Custom software developed by DRES and DPG were used to analyze slit plates. The Colony Image Analysis (CIA) software locates and analyzes each colony and provides the plate and sample count. Data output in Excel format can be further processed by conventional software. Statistical analysis was done with SigmaStat version 2 (SPSS Inc. 444 N. Michigan Avenue, Chicago, Illinois, 60611).

RESULTS AND DISCUSSION

Aerosol measurements, unlike analytical chemistry determinations, are subject to great numerical variances influenced by a variety of parameters that are difficult to control. Slight variations in experimental maneuvers can contribute to different results that may appear unacceptable. For this series of chamber experiments, we structured an approach that would account for variations such as the act of weighing the starting material. It can be seen that overall, larger amounts of dry BG material, when rendered into suspension, gave correspondingly greater viable counts in each replicate experiment (Table 1). When the mean viable counts of the replicate are compared, Figure 1 shows that there is a log-linear relationship with respect to dry weight.

Table 1. Summary of Suspension Concentrations from Different starting amounts of dry BG powder. Slightly different viable counts reflect handling

Dry Weight/ml Viable spores/ml	5 μg	10 μg	20 μg	30 μg
1	1.64E+06	1.14E+06	1.91E+06	4.12E+06
2	7.35E+05	1.35E+06	1.37E+06	5.22E+06
3	7.41E+05	1.22E+06	3.67E+06	5.09E+06
4	6.66E+05	1.23E+06	3.04E+06	5.11E+06
5	6.84E+05	1.27E+06	3.13E+06	4.33E+06
6			2.80E+06	5.74E+06
7			1.91E+06	
Mean	8.93E+05	1.24E+06	2.55E+06	4.94E+06

Comparing the mean values of viable counts is a valid approach because the raw determinations passed normality test ($P > 0.200$) and equal variance test ($P = 0.062$). It can be seen that although the mean viable spores/ml value from the 5 μg

batch appeared to be lower than that from the 10 μg, Tukey's multiple means test showed no significant difference. This could be a reflection of the difficulty in accurately weighing out 0.05 gm dry powder material for each replicate test. A comparison of the respective SEM or standard error of the mean (187254 for 5 μg vs 34263 for the 10 μg data sets) revealed greater inconsistencies during weighing material in less than 0.1 mg batches using a Mettler balance. This observation, suggests that dry weight is not a very reliable reference. Instead, suspension concentration, expressed as viable spores/ml is more reliable and has been used as the reference for each replicated experiment.

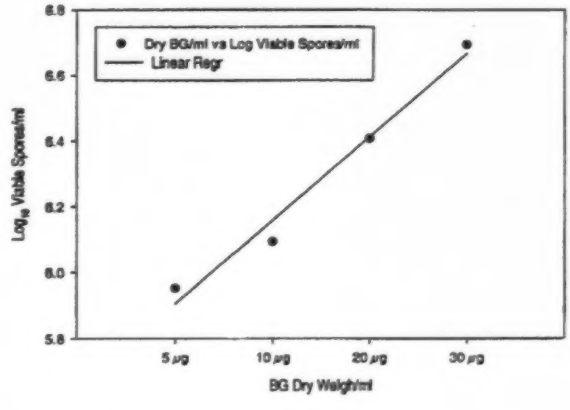

Figure 1. Plot of dry BG powder by weight vs mean log viable spores in one ml of liquid suspension. Each experimental run starts with the weighing of dry BG powder to take into account weighing errors that may affect final outcome. In practice, 50 to 100 mg batches of the material were deemed appropriate for weighing accuracy. Working suspensions were serially diluted to the final concentrations that are used in the sprayer. Viable spore counts were performed on the final preparation.

Aerosol characterization with slit samplers.

After about 15 minutes from aerosol generator startup, equilibration of the aerosol concentration was achieved, as indicated by on/off cycling of the software controlled switcher. Slit sampling sequence was initiated via a remote control that activated the first in a series of 10 devices. Simultaneously, the vacuum source was also activate providing the desired air flow. With each sample plate revolving at 1 rpm, this device provides a very fine resolution of the instantaneous aerosol concentration, much more so than all the other devices. When the resultant colony counts were analyzed for the 30 segments per plate, each representing a 2 second time slice, the 300 data points can be depicted as a frequency distribution plot,

shown in Figure 2. Each data set (n = 300) when subjected to normal distribution testing, failed without exception. This confirms similar observation for field trial measurements of biological aerosol using optical methods (Ho et al. 1999). Tillett and Carpenter (1991) also reported that epidemiological data in microbiology were not normally distributed, thus recommending the use of non-parametric statistical techniques.

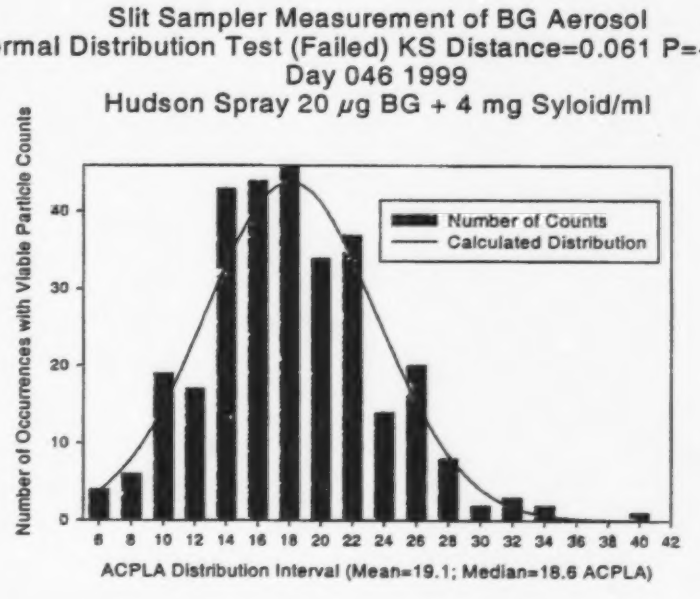
Slit Sampler Measurement of BG Aerosol
Normal Distribution Test (Failed) KS Distance=0.061 P=<0.010
Day 046 1999
Hudson Spray 20 µg BG + 4 mg Syloid/ml
Number of Occurrences with Viable Particle Counts
Number of Counts
Calculated Distribution
0
10
20
30
40
6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42
ACPLA Distribution Interval (Mean=19.1; Median=18.6 ACPLA)

Figure 2. Typical normal distribution analysis of slit sampler data from a 10 min run. The chamber was filled with the desired BG spore aerosol using a Hudson generator. After equilibration of the aerosol concentration at a preset level, the first of 10 serially connected slit samplers was started. Flow rate had been calibrated for 50 L/min. To ensure uniform performance for each sampler, the same vacuum source was applied to each device by using a switched line controlled by the built-in solenoid valve synchronized to the turn table on/off signal. petri plates were incubated overnight and counted.

So instead of obtaining the mean over the 10 minute sampling period, the appropriate way is to report the median aerosol concentration, expressed in agent containing particles per liter of air (ACPLA). In Figure 2, the median of 18.6 was mathematically derived by the software that performed the distribution calculation. Numerically this was slightly lower than the estimated mean (19.1). For consistency and statistically correctness, all subsequent slit sampler data are reported as median ACPLA measured over the sampling period.

Figure 2 gives a very good illustration of how biological aerosol sampling is best described as a population study where at any given time, the actual concentration can be anywhere between two extremes of the distribution. In this example,

there were samples that registered in the low end at 6 while others were in the high at 34 ACPLA. To fully describe biological aerosol characteristics, we recommend that these descriptive treatments should represent minimal requirements.

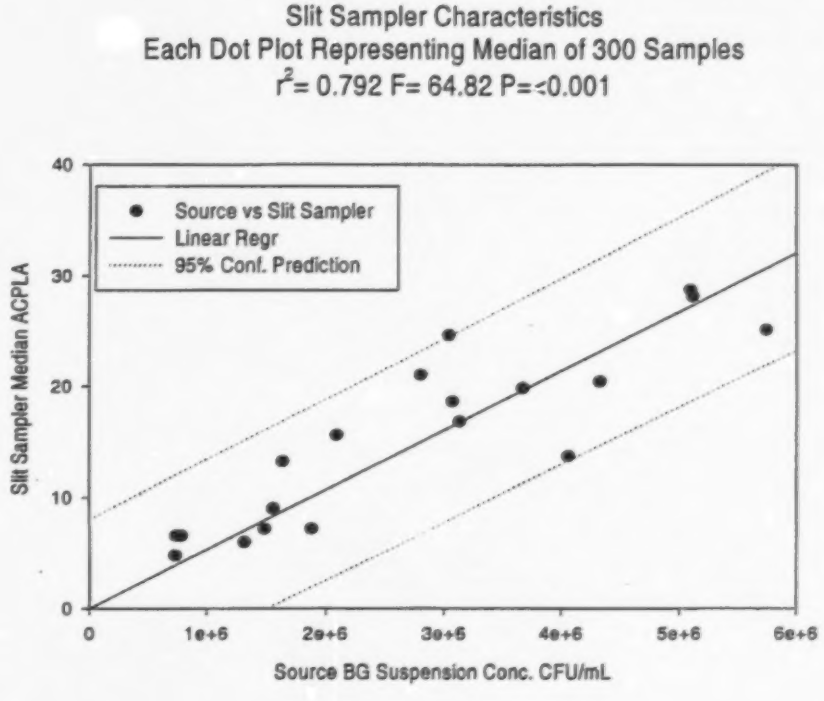

Figure 3. Cumulative comparison of slit sampler data from multiple tests with different starting BG concentrations. Spore suspensions (BG) of known viable concentration were used to produce aerosol with a Hudson generator. Slit samplers collected viable particles on petri plates with a 2 second time resolution. Median values from 300 data points were used to depict each determination. Results shown here representing work done over a 6 month period in a 90 m^3 chamber suggest that it is possible to select and maintain a desired aerosol concentration to perform useful work.

Each experimental run, using a known concentration of spore suspension, provides one data point that is a statistical representation of the viable particle concentration, expressed as the median ACPLA. When the suspension strength was changed, one would expect the median ACPLA to behave correspondingly and the results are summarised in Figure 3. Given the complexity of the data handling process, we were gratified to see that median ACPLA values appear to increase with increasing suspension concentration. Moreover, when the data points were fitted to a linear regression routine, a respectable coefficient of 0.792 resulted, with the extrapolated curve passing through the origin. Another useful observation was that

most of these points were within the 95% confidence boundary. By inspecting the curve, it can be seen that a number of experiments yielded data points close to the 5 ACPLA mark, suggesting that it may be possible to repeatedly establish such low biological aerosol concentration in the chamber. Indeed, Figure 3 provides useful information as the starting point to permit the user to select a desired ACPLA level for detector challenge work. By picking an appropriate suspension concentration, there is reasonable probability that the desire median APCLA can be obtained.

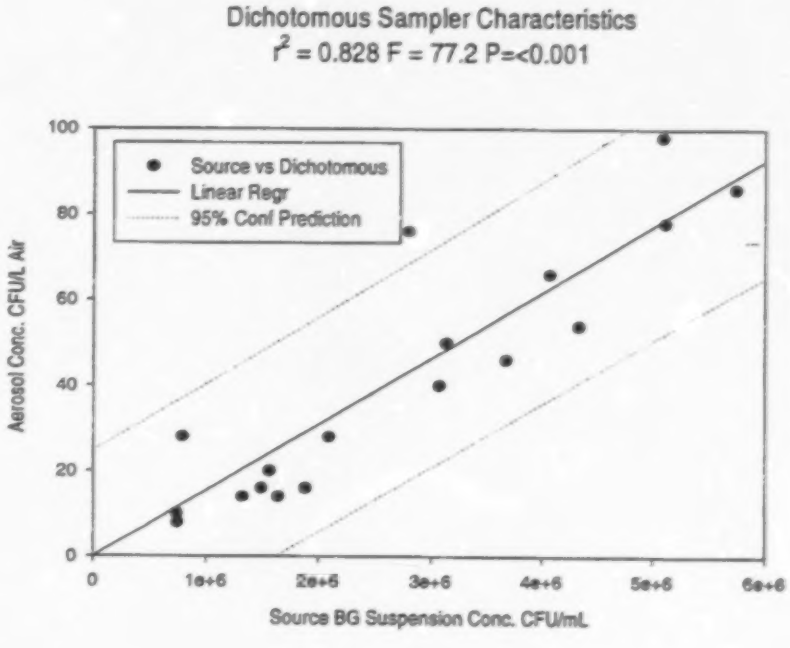

Figure 4. Cumulative comparison of Dichotomous sampler data from multiple test with different starting BG concentrations. A dichotomous sampler collects particles on a pair of glass fiber filters with 2.5 μm cutoff. Each filter was disrupted with a wrist action shaker in 20 ml distilled water and the supernatant was assayed for viable spores. For this report, the data from both filters have been normalised to CFU/L then summed to give a single representation of the aerosol concentration in the test chamber. Actually sampling was done at 20 minute intervals and each data point shown here represents an average from 3 replicates.

While the slit samplers were running, other aerosol samplers were also activated at about the same time. Figure 4 shows the results of measuring viable single spores expressed as number counts (colony forming units, CFU) per liter of air with the dichotomous sampler. Spore particles were trapped on glass fiber filters and the viable population was assessed as described in methods. It has been frequently asked if violent mechanical action is needed to ensure that the individual

spores are represented, for example sonication might be used to dislodge the last few aggregates. In a previous study (Ho and Fisher, 1993), it was shown that sonication increased viable counts in a BG spore suspension by a factor of 1.72. In the same sample, electronically counting total particles greatly increased that by a factor of 5.43. In contrast, by microscopic counts, the increase was only by a factor of 3.94. It is clear that extremely vigorous mechanical disruption did improve the individual viable counts though not as dramatically as electronic particle counts. The benefit must be balanced in instances where many samples need be processed. The extra effort to gain less than a factor of two, a minor benefit, may not be justified. We suggest that the "wrist action shaker" method provides adequate disruption yet providing acceptable performance.

It has been mentioned that biological aerosol particles may consist of aggregates (Ho, 1994). Given that DS assessment revealed individual viable spore counts while the slit samplers yielded viable particle counts, it stands to reason that under similar conditions, the former value would always be numerically larger. By inspecting the respective values, normalised to per unit aerosol volume in Figures 3 and 4 (compare respective Y axis, with X axis unchanged), it can be seen that the DS values are higher than those from the slit samplers. Obviously, for this comparison to be meaningful, some assumptions would have to be made with respect to their respective instrument sampling efficiencies.

Upton et al. (1994) determined sampling efficieny for the Casella slit sampler, a design related to the New Brunswick instrument. They reported values of >80% for 2.5 μm fluorescein particles. A lower value was found for 5 μm particles (50%) and they attributed this to poor inlet design. We agree with their assessment; differences in the inlet designs of these two samplers are shown in the standard aerosol handbook (Herring, 1989). It can be seen that the Casella inlet is much narrower in diameter yet taller in height compared to that of the New Brunswick. Thus, the latter design may perform better. Another possible source of error may be their unfortunate choice of collection medium (Upton et al., 1994). To capture particles, glass fiber filters were used instead of standard 15 cm diameter petri dishes with agar, the intended impaction medium. These factors together with their use of non-biological test particles render their efficiency results less helpful in deducing slit sampler efficiency. Without any other convenient references, it may be reasonable to suggest 70-80% as the New Brunswick sampling efficiency.

The reference sampler used by Upton et al. (1994) was an isokinetic probe attached to glass fiber filter running at unspecified flow rates. This is functionally equivalent to our use of the DS, except in this case, the sample stream has been size separated by a virtual impactor (McFarland et al. 1978) with separation at 2.5 μm. Analysis of DS results (Table 2) from the experiments revealed that average viable spore from each size group was 28% for fine (<2.5 μm) and 72% for coarse (>2.5 μm). This observation is of course valid only for the Hudson generator used throughout these tests. It has been shown that different sprayer designs have significant influence on fractional distribution, biased to the coarse side for devices that produced larger liquid droplets like the Micronair agricultural sprayer (Ho, 1991).

Table 2. Descriptive Statistics of Dichotomous Sampler Data

Column	Size	Missing	Mean	Std Dev	Std. Error	C.I. of Mean
Fine%	68	0	28.243	13.756	1.668	3.330
Course%	68	0	71.757	13.756	1.668	3.330

Column	Range	Max	Min	Median	25%	75%
Fine%	51.006	52.824	1.818	31.411	18.182	38.838
Course%	51.006	98.182	47.176	68.589	61.162	81.818

Column	Skewness	Kurtosis	K-S Dist.	K-S Prob.		Sum	Sum of Squares
Fine%	-0.332		-0.932	0.104	0.067	1920.508	66918.592
Course%	0.332		-0.932	0.104	0.067	4879.492	362817.090

Normality Test (Kolmogorov-Smirnov)

Fine%:	K-S Dist. = 0.104	P = 0.067	Passed
Course%:	K-S Dist. = 0.104	P = 0.067	Passed

Establishing sampling efficiencies is a difficult task because there is no easy way to precisely assess the real content in a biological aerosol. Unlike inanimate aerosols like fluorescein particles or latex beads that can be assessed by a variety of ways, (e.g. weighing or fluorescence measurement), biological material must be shown to be alive. Lacking a definitive approach, one alternative is to use other "standard" samplers to obtain comparative data. For this reason, the all glass impinger (AGI) has been used to provide numbers to help with putting all the analyses in perspective. Chatigny et al. (page 213, 1989) stated that for *Bacillus subtilis*, spore recovery is approximately equal in the AGI and the slit sampler.

Figure 5 shows the cumulative AGI results presented in the same format as the previous ones. All the data points conformed to a linear regression fit with the extrapolated curve passing through the origin. The regression coefficient (0.736) is comparable to that from the previous tests. This high value reflects little data point scatter and suggests statistically reproducible measurements. A newly designed glass impinger (SKC) was also used during the later part of this work. Willeke et al. (1998) explained that the new nozzle design minimized reaerosolization, possibly resulting in efficiency values of 95-100%.

The SKC results are presented in Figure 6 as linear regression plot, in direct comparison with the various devices. Apart from the slit sampler data that are referenced to the right hand Y axis, the other instruments reflect the same aerosol concentration in viable individual spores per unit volume. It can be seen that the SKC sampler measurements appeared to be higher than those of the standard AGI. However, the low regression coefficient suggests that it was more difficult to obtain reproducible results with the SKC sampler.

The lesson learned from inspecting Figure 6 is that both the glass impingers appear to be inherently less efficient than collecting aerosol material on filters, the medium employed in the DS (Table 3). For estimation of relative sampling efficiencies, the slopes of each curve (AGI and SKC) were divided by that from the DS curve multiplied by 100. With its high recovery performance and reproducibility, the DS has all the characteristics the best reference sampler for efficiency studies.

Table 3. Comparison of Relative Sampling Efficiencies

	Slope	% Eff ref DS
AGI	4.17E-06	26.97
SKC	6.98E-06	45.16
DS	1.55E-05	

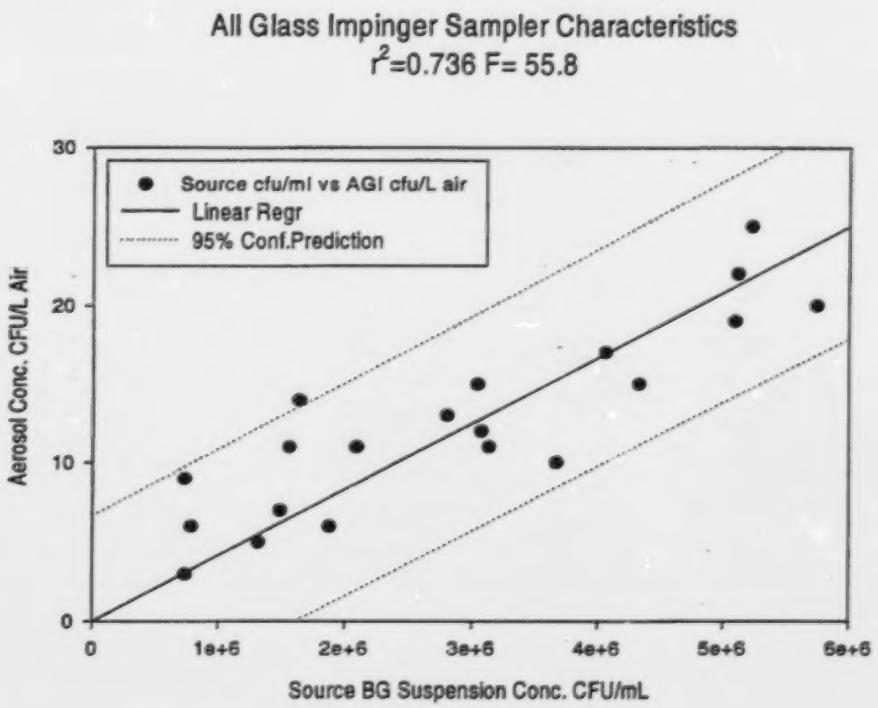

Figure 5. Cumulative comparison of AGI sampler data from multiple test with different starting BG concentrations.A series of 5 minute sample replicates were taken with standard glass impingers using 20 ml distilled water collection fluid. Conventional viable assays were performed using Spiral Plating techniques on nutrient agar. Each data point represents an average of 11 replicates. Similar sampling regime was applied to the SKC except there were only 9 replicates.

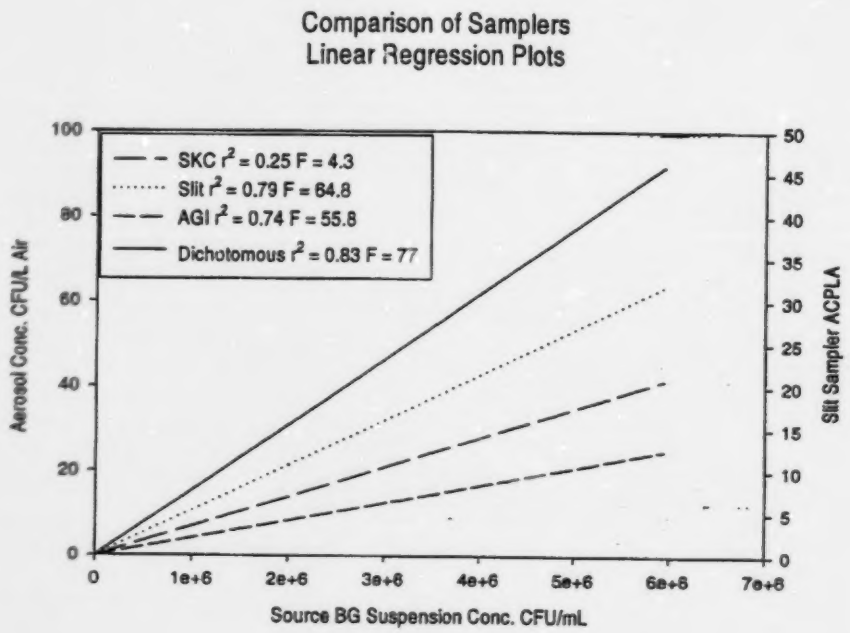

Figure 6. Summary of linear regression statistics describing collection characteristics of common biological aerosol samplers. The slope of the r^2 regression line for each sampler system is compared to give an indication of their relative efficiency. An indication of how well each sampler performed with respect to reproducibility is reflected in how close the r^2 value is to unity. The F statistic is a measure of tendency towards randomness or variance. Larger F numbers indicate smaller variance. Of the samplers examines, the SKC appeared to collect more efficiently than the AGI but in reality, it suffered from inconsistency. To be fair, this sampler was a recent addition to the array and consequently does not have as many cumulative data points (n = 14 vs 21). Note that the slit sampler has a different Y axis scale (right side) reflecting a measurement of viable particles as opposed to individual spores.

As stated previously, each experiment using a known suspension yielded a pair of data points that described the particle concentration as well as the viable spore content per unit volume. Many replicate runs were done to provide a series of data points plotted along an X/Y axis. This exercise revealed a curve exhibiting linear relationship with 0.816 regression coefficient (Figure 7). Data scatter appears to fall within the 95% confidence prediction boundary.

A straight line curve is defined by the simple equation Y=a+bX. When applied to this observation, units of measure can be linked to the variables as shown:

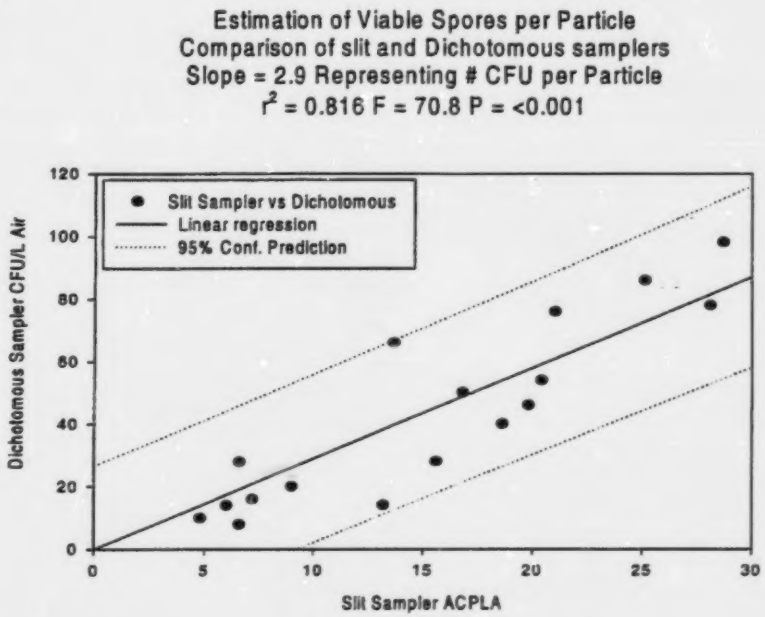

Figure 7. A suspension of BG spores of known concentration in a Hudson nebulizer was used as starting material to generate biological aerosol in a 90 m^3 chamber. Once the aerosol concentration has stabilized, replicate samples of the particles in air were taken with a New Brunswick slit sampler (300). A dichotomous sampler measured CFU/L in air (3 replicates). The results from each device were averaged and each point on the plot represents one experimental run. By varying the viable spore suspension concentration in each experiment, a series of aerosol measurements were obtained from the sampler pair. Since the slit sampler measured particles in a liter of air and the dichotomous sampler determined CFU/L, the linear regression plot of the data sets provides a slope that is equivalent to the average number of spores bound in each particle.

Y = dichotomous sampler in viable spores/ liter air
X = slit sampler in viable particles/ liter air
a = intercept which is zero since the curve passes through the origin
b = slope of the curve

Rearranging this equation :

b = Y/X or viable spore/viable particles after canceling the unit volume

The resulting slope leads to the key component:
b = viable spore per particle

As seen in Figure 7, the slope calculation was 2.9, the average number of viable spores per particle.

Obviously, as a mathematically derived value, it may only reflect a crude representation of reality. In table 2, it has been suggested that about 28% of the population at any given time were <2.5 μm in size. In this small volume, it is unlikely that it can contain more than one viable spore and the rest of the volume was probably taken up by extracellular material. Also, the slit sampler efficiency may be adjusted downwards to account for some sampling inefficiencies for particles >2.5 μm. The sum of these corrections may help boost "b" by about 50%. We therefore propose, for a particle >2.5μm, a new value as 4.5 spores per particle.

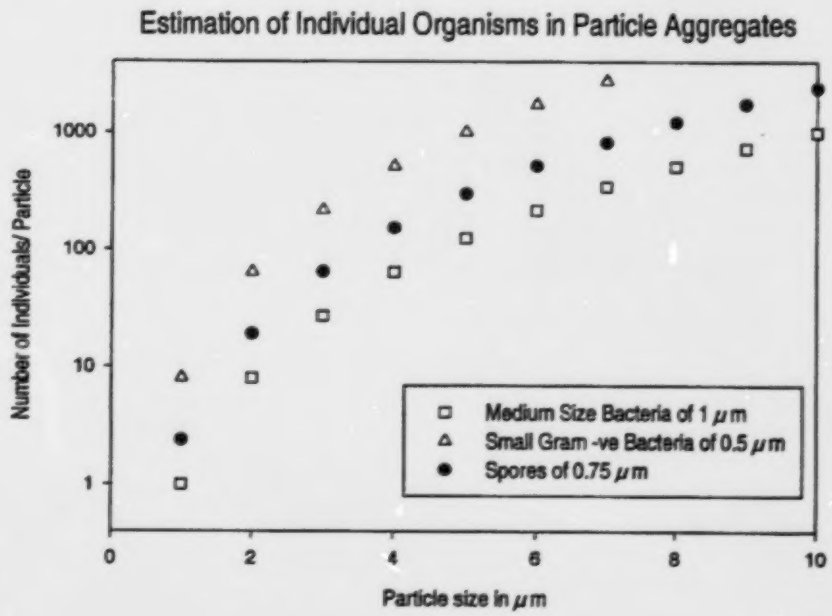

Figure 8. Mathematical exercise to demonstrate the number of different size bacteria that can appear in aggregates of various diameters. The model is based on theoretically clean organisms without extracellular material that otherwise may also take up more space.

This value can be tested by an empirical demonstration to estimate the number of single cells within a larger aggregate as shown in Figure 8. It describes an attempt to estimate how many organisms of a specified diameter can be made to fit within a larger aggregate by comparing volumetric ratios. To show how this model can explain the current problem, consider a 4 μm spore aggregate. By inspecting the spore model in Figure 8, it can be seen that slightly over 100 individuals can fit into this space. Only about 10% of the particles in the working BG suspension was pre-

viously shown to be viable (Ho and Fisher, 1993). With this knowledge, the corrected hypothetical 4 μm particle will contain about 10 viable spores.

Microorganisms that can survive in nature's harsh environment have evolved protective mechanisms. *Bacillus subtilis* can produce levan, a fructose polymer, in direct proportion to sugar content in the growth medium (Elisashvili, 1984). *Erwinia herbicola* can reportedly produce up to 19% by weight in levan under optimal growth conditions (Keith et al., 1991). For the present discussion, as correction, we can apply 20% by weight to the BG preparation to account for non-cell related material like levan, growth media and other unknowns. The revised number now becomes 8 viable spores per particle as summarised in Table 4.

Table 4. Summary of Correction Factors Applied to a 4 μm Particle

	Estimation
Corrections applied	Number of individuals
Native particles predicted in Fig. 8	~100
10% viability (Ho and Fisher, 1993)	~10
19% levan + 1% growth medium	~8
Proposed by this paper by measurement	4.5

In final analysis, the proposed value of 4.5 viable spores in a typical particle of >2.5 μm is not unreasonable. Its true diameter may never be known but by projection, it should fall between 2.5 to 4 μm by these calculations. Coincidentally, this size range fits in well with what Day (1981) has considered as critical for potential biological threat aerosols.

CONCLUSIONS

By using the existing DRES 90 m^3 chamber, it has been shown that low BG aerosol concentrations (5 ACPLA) can be generated reproducibly. This will be a useful feature for biological detector testing. Particle collection characteristics of the slit sampler have been studied. This is the modified version used in international refereed field trails. A statistical method was used to demonstrate procedural predictability at 95% confidence level. By using glass fiber filters mounted in a dichotomous sampler, sampling efficiencies were estimated for a variety of common aerosol collectors. This led to the selection of glass fiber filter as the optimal reference collector medium for comparative efficiency studies. The accumulated data permitted the estimation of the number of viable spores per particle. For a 2.5 to 4 μm particle, arguments have been presented for considering 4.5 as the most probable value. This is a useful conversion factor to assist comprehension of ACPLA data.

One caveat is that this value relates only to the Hudson nebulizer. The intent has been that once the proposed method is established and found acceptable for such studies, similar work can be done to characterize other generators and indeed other biological material.

Conventional aerosol facilities utilise continuous particle generators that produce a point source challenge to detectors or collectors (Keith et al., 1991). With

this approach aerosol delivery at the receiving end is an inherently random process and these authors have acknowledged the problem by attempting to solve it with distribution grids. We have described an alternative approach; by filling a chamber with a constant concentration of particulate material, replicate sampling is now a feature that has been missing from other designs. The results obtained from multiple sampling permit statistical analysis to be performed. Reliable analytical results can lead to interesting predictions, for example, estimation of viable spore numbers in a particle.

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APPENDIX 1

Many workers outside the field of microbiology are confused with the terms "culturable, viable and viable but non-culturable" when applied to bacteria physiology. It used to be a simple distinction that when a cell is viable, then it can be grown or cultured on artificial media. But many workers have pointed out that there are instances where the correct growth media has not been developed of some organisms that are either naturally occurring or injured from environmental exposure. It is beyond the scope of this paper to start a discussion on the definition of "viable but non-culturable" bacteria. There are many reports that claimed to have the ability to measure this non-culturable characteristic in an assortment of bacteria (attached below). On the other hand, there are also those that believe the proper control experiments have not been done to distinguish the various states of viability (Kell et al. 1998). In this paper, we use the terms live, viable and culturable interchangeably to describe organisms that can replicate in basic nutrient growth media and form visible colonies, also known as colony forming units (CFU). Included below are some recent papers that describe the current state of research in this debate. They are here to provide a guide to those new to the controversy.

Sunlight and the survival of enteric bacteria in natural waters. Davies CM, Evison LM J Appl Bacteriol 1991 Mar 70:3 265-74

Abstract: Escherichia coli and some salmonellas were exposed in seawater and freshwater to natural sunlight, visible light of comparable intensity, and light containing a similar proportion of u.v. as natural sunlight but of a much lower intensity. Direct viable bacterial counts and culturable counts on selective and non-selective media were made at intervals. The rate of decrease in numbers of culturable bacteria was significantly faster in seawater than in freshwater when exposed to natural sunlight. No significant difference was found between the rates of decrease in numbers of culturable bacteria in seawater and those in freshwater when bacteria were exposed to light with a small u.v. component of similar intensity. The effect of salinity no loss of culturability is, therefore, more sig-nificant in the presence of u.v. radiation. Direct counts by the acridine orange direct viable count method decreased much more slowly than the culturable counts in sea-water but comparably with culturable counts in freshwater in natural sunlight. Direct viable counts and culturable counts decreased at a similar rate in seawater and in freshwater in visible light. This may signify the evolution of enteric bacteria towards a viable but non-culturable form in seawater when exposed to natural sunlight. The presence of humic acids significantly reduced loss of culturability but only in low salinity conditions. Salinity appears to be an important factor influencing culturabil-ity in bacteria exposed to sunlight.

Department of Civil Engineering, University of Newcastle upon Tyne, UK.

Workers' exposure to airborne bacteria and endotoxins at industrial wastewater treatment plants. Laitinen S, Kangas J, Kotimaa M, Liesivuori J, Martikainen PJ, Nevalainen A, Sarantila R, Husman K Am Ind Hyg Assoc J 1994 Nov 55:11 1055-60

Abstract: A study of sewage workers' exposure to airborne culturable bacteria and inhaled endotoxins was performed at nine waste-water treatment plants that treat mainly industrial effluents. Airborne endotoxins were collected on glass fiber filters and analyzed using a chromogenic limulus assay. Endotoxin concentrations measured in the immediate vicinity of the waste-water treatment process varied from 0.1 to 350 ng/m3. The eight-hour time weighted average concentrations of endotoxin to which workers were exposed exceeded the suggested exposure limit (30 ng/m3 endotoxin) at four of the plants. Air samples of culturable bacteria concentrations varied between 10 and 10(5) colony-forming units/m3. Of the particles carrying culturable bacteria, 88% had an aerodynamic diameter of less than 4.7 microns. The most common genera of airborne gram-negative bacteria were acinetobacter, citrobacter, enterobacter, klebsiella, and pseudomonas. High levels of exposure to bacteria and bacterial endotoxin usually were related to certain phases of the treatment process. The microbiological contamination of air was highest near the inlets where in-

coming wastewater entered the basins, in the sludge treatment area, and inside the biofilter tower. In these spaces it is necessary to control and re-duce exposure to airborne bacteria and endotoxin at wastewater plants.

Kuopio Regional Institute of Occupational Health, Finland.

Total counts, culturable and viable, and non-culturable microflora of a French min-eral water: a case study. Defives C, Guyard S, Oularé MM, Mary P, Homez JP J Appl Microbiol 1999 Jun 86:6 1033-8

Abstract: The changes in bacterial counts during the storage of a natural mineral water from a French spring were studied. Samples were taken from the spring and the bottling line. Viable cultivable (VC) bacteria were counted on R2A medium. Total counts, viable and dead bacteria were counted using the LIVE/DEAD Bac Light VIABILITY kit and epifluorescence microscopy. Viable but non-cultivable (VNC) bacteria were estimated by difference between viable and VC counts. Isolates were clustered by phenotype. The microflora in the spring water increased from < 10-3 x 10(5) bacteria ml-1 after 6 d in storage and then stabilized. Mechanical bottling increased the allochthonous bacteria in the water that stabilized at 10(5) bacteria ml-1. Maximal growth is controlled by the low concentration of nutrients in the mineral water and the lysis of dead cells. The allochthonous bacteria came from the aquifer and colonized the filling line. The changes in the VC and VNC populations showed that the bacteria used starvation-survival and entry into the VNC state to adapt to the bottling stress and the enclosed oligotrophic environment.

Laboratoire de Microbiologie Fondamentale et Appliquée, Université des Sciences et Technologies de Lille, Villeneuve d'Ascq, France.

Double staining (CTC-DAPI) for detection and enumeration of viable but non-culturable Campylobacter jejuni cells. Cappelier JM, Lazaro B, Rossero A, Fernandez-Astorga A, Federighi M Vet Res 1997 Nov-Dec 28:6 547-55

Abstract: Direct microscopic enumeration of viable Campylobacter jejuni cells (ie, respiring bacteria) were performed in both culturable and non-culturable states. Five different C jejuni strains were used, including a reference strain, ATCC 33291. Cells from all five strains were incubated alone with 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), a redox dye. It was reduced by an electron transport chain to an insoluble red fluorescent CTC formazan salt, which accumulated intracellularly. The presence of these red CTC crystals in the bacteria cells was indicative of cellular respiratory activity. Counterstaining with 4'-6 diamino-2 phenylindole (DAPI), which fluoresces in blue, made a suitable contrast and allowed simultaneous enumeration of total and viable bacteria on a single filter. Four hours of incubation with 5 mM CTC under a microaerobic atmosphere was found to be the optimal condition yielding the maximum number of respiring cells (both culturable and non-culturable). When used in combination with standard culture techniques, double staining makes it possible to monitor the viable but non-culturable cells of jejuni obtained by starvation more easily than with the direct viable count procedure.

Ecole nationale vétérinaire de Nantes, unité associée Inra d'hygiène alimentaire, France.

[Resuscitation from the viable but nonculturable state of Helicobacter pylori] Kurokawa M, Nukina M, Nakanishi H, Tomita S, Tamura T, Shimoyama T Kansen-shogaku Zasshi 1999 Jan 73:1 15-9

Abstract: It is well known that the change of state from the culturable to not culturable on pathogenic organisms could easily occurred. These states of bacteria were so called viable but nonculturable (VNC). The coccoid form of H. pylori is seems to be in this state. The possibility of resuscitation of H. pylori was examined in vitro. The coccoid form bacteria was treated with Ammonium Sulfate and heat shock before culture, and cultured in Brucella broth containing, sodium pyruvate, laked human erythrocyte and serum. The growth of the

rod spiral form was found in the coccoid form bacteria, and further the bacteria could grow on blood agar and Skirrow agar as well as the original strain. It was strongly suggested that the resuscitation from VNC state of cells to culturable form consists of two processes, stimulation and supplemented of appropriate nutrition.

Department of Bacteriology, Kobe Institute of Health.

Isolation of new bacterial species from drinking water biofilms and proof of their in situ dominance with highly specific 16S rRNA probes. Kalmbach S, Manz W, Szewzyk U Appl Environ Microbiol 1997 Nov 63:11 4164-70

Abstract: A polyphasic approach involving cultivation, direct viable counts, rRNA-based phylogenetic classification, and in situ probing was applied for the characterization of the dominant microbial population in a municipal drinking water distribution system. A total of 234 bacterial strains cultivated on R2A medium were screened for bacteria affiliated with the in situ dominating beta subclass of Proteobacteria. The isolates were grouped according to common features of their cell and colony morphologies, and eight representative strains were used for 16S rRNA sequencing and the development of a suite of strain-specific oligonucleotide probes. Phylogenetic analysis indicated that all of the isolates were hitherto unknown bacteria. Three of them, strains B4, B6, and B8, formed a separate cluster of closely related organisms within the beta 1 subclass of Proteobacteria. In situ probing revealed that (i) 67 to 72% of total bacteria, corresponding to more than 80% of beta-subclass bacteria, could be encompassed with the strain-specific probes and (ii) the dominating bacterial species were culturable on R2A medium. Additionally, two-thirds of the autochthonous drinking water population could be shown to be in a viable but nonculturable (VBNC) state by using a direct viable count approach. The comparison of isolation frequencies with the in situ abundances of the eight investigated strains revealed differences in their culturability, indicating variable ratios of culturable to VBNC cells among the strains. The further characterization of biofilms throughout the distribution network demonstrated strains B6 and B8 to be dominant bacterial strains in groundwater and distribution system biofilms. The other strains could be found at various frequencies in the different parts of the distribution system; several strains appeared exclusively in drinking water biofilms obtained from a house installation system.

Fachgebiet Okologie der Mikroorganismen, Institut für Technischen Umweltschutz, Technische Universität Berlin, Germany.

Luminescence-based detection of activity of starved and viable but nonculturable bacteria. Duncan S, Glover LA, Killham K, Prosser JI Appl Environ Microbiol 1994 Apr 60:4 1308-16

Abstract: A naturally luminescent bacterium, Vibrio harveyi, and two bacteria, Escherichia coli and Pseudomonas fluorescens, which had been genetically marked with luminescence were starved in liquid medium at 4 and 30 degrees C for 54 days. Total cell concentrations and concentrations of culturable and viable cells were determined by acridine orange staining, dilution plate counting, and direct viable counting, respectively, and population activity was measured by luminometry. V. harveyi became nonculturable but maintained viability during starvation at 4 degrees C and maintained both culturability and viability at 30 degrees C. In contrast, E. coli became viable but nonculturable during starvation at 30 degrees C but not at 4 degrees C. Luminescence of nonculturable cells of both strains, and culturable cells of V. harveyi, decreased to background levels during starvation. Luminescence of starved culturable cells of E. coli also fell below background levels but occasionally increased to detectable values. Viable, nonculturable forms of P. fluorescens were not detected at either temperature, and cells starved at 4 degrees C showed no decrease in luminescence measured during incubation of samples at 25 degrees C. Following incubation of late-log-phase cells with yeast extract and nalidixic acid, changes in light output directly paralleled changes in cell length, as observed during direct viable counting. Quantification of changes in luminescence following incubation of starved cells with yeast extract enabled measurement of the activity of both culturable and viable but nonculturable cells.

Measurement of luminescence was significantly more sensitive, rapid, and convenient in quantifying activity following nutrient amendment than measurement of changes in cell length.

Detection of non-culturable Shigella dysenteriae 1 from artificially contaminated volunteers' fingers using fluorescent antibody and PCR techniques. Islam MS, Hossain MZ, Khan SI, Felsenstein A, Sack RB, Albert MJ J Diarrhoeal Dis Res 1997 Jun 15:2 65-70

Abstract: Epidemiological studies have demonstrated that hands may be an important vehicle for transmission of shigellosis. The present study was carried out to find out the survival potential of Shigella dysenteriae 1 on fingers of volunteers. Finger surface was inoculated with 10(5) cfu of S. dysenteriae 1 and then the bacteria were detected using conventional culture, PCR and fluorescent antibody (FA) techniques after different time intervals. It was found that S. dysenteriae 1 survived for up to one hour in culturable form but up to four hours in non-culturable form on human fingers. The non-culturable S. dysenteriae was detected by PCR and FA techniques. This study elaborates on the role that fingers have in the transmission of shigellae.

International Centre for Diarrhoeal Disease Research, Bangladesh, Dhaka, Bangladesh.

Comparison of methods for detection and enumeration of airborne microorganisms collected by liquid impingement. Terzieva S, Donnelly J, Ulevicius V, Grinshpun SA, Willeke K, Stelma GN, Brenner KP Appl Environ Microbiol 1996 Jul 62:7 2264-72

Abstract Bacterial agents and cell components can be spread as bioaerosols, producing infections and asthmatic problems. This study compares four methods for the detection and enumeration of aerosolized bacteria collected in an AGI-30 impinger. Changes in the total and viable concentrations of Pseudomonas fluorescens in the collection fluid with respect to time of impingement were determined. Two direct microscopic methods (acridine orange and BacLight) and aerodynamic aerosol-size spectrometry (Aerosizer) were employed to measure the total bacterial cell concentrations in the impinger collection fluid and the air, respectively. These data were compared with plate counts on selective (MacConkey agar) and nonselective (Trypticase soy agar) media, and the percentages of culturable cells in the collection fluid and the bacterial injury response to the impingement process were determined. The bacterial collection rate was found to be relatively unchanged during 60 min of impingement. The aerosol measurements indicated an increased amount of cell fragments upstream of the impinger due to continuous bacterial nebulization. Some of the bacterial clusters, present in the air upstream of the impinger, deagglomerated during impingement, thus increasing the total bacterial count by both direct microscopic methods. The BacLight staining technique was also used to determine the changes in viable bacterial concentration during the impingement process. The percentage of viable bacteria, determined as a ratio of BacLight live to total counts was only 20% after 60 min of sampling. High counts on Trypticase soy agar indicated that most of the injured cells could recover. On the other hand, the counts from the MacConkey agar were very low, indicating that most of the cells were structurally damaged in the impinger. The comparison of data on the percentage of injured bacteria obtained by the traditional plate count with the data on percentage of nonviable bacteria obtained by the BacLight method showed good agreement.

Department of Environmental Health, University of Cincinnati, Ohio-45267-0056, USA.

Non-culturable Legionella pneumophila associated with Acanthamoeba castellanii: detection of the bacterium using DNA amplification and hybridization. Hay J, Seal DV, Billcliffe B, Freer JH J Appl Bacteriol 1995 Jan 78:1 61-5

Abstract The intracellular localization of Legionella pneumophila serogroup 1 within Acanthamoeba castellanii rendered the bacteria non-culturable on supplemented BCYE agar. DNA amplification, using two 19-mer primers, and hybridization using a 25-mer oligonucleotide probe, permitted detection of Leg. pneumophila in approximately 81% (29/36) of

samples where the bacteria could not be detected using culture. A combination of co-cultivation of samples with Leg. pneumophila-naive A. polyphaga or Hartmannella vermiformis, incubation in a defined liquid medium or use of catalase indicated that approximately 31% (9/29) of the samples contained Leg. pneumophila which were viable although not culturable.

Department of Ophthalmology, University of Glasgow, UK.

Isolation and characterization of full-length chromosomes from non-culturable plant-pathogenic Mycoplasma-like organisms. Neimark H, Kirkpatrick BC Mol Microbiol 1993 Jan 7:1 21-8

Abstract We describe the isolation and characterization of full-length chromosomes from non-culturable plant-pathogenic, mycoplasma-like organisms (MLOs). MLO chromosomes are circular and their sizes (640 to 1185 kbp) are heterogeneous. Divergence in the range of chromosome sizes is apparent between MLOs in the two major MLO disease groups, and chromosome size polymorphism occurs among some related agents. MLO chromosome sizes overlap those of culturable mycoplasmas; consequently, small genome size alone cannot explain MLO non-culturability. Hybridization with cloned MLO-specific chromosomal and 16S rRNA probes detected two separate chromosomes in some MLO 'type' strains. Large DNA molecules that appear to be MLO megaplasmids were also demonstrated. The ability to characterize full-length chromosomes from virtually any non-culturable prokaryote should greatly facilitate the molecular and genetic analysis of these difficult bacteria.

Department of Microbiology and Immunology, Morse Institute for Molecular Biology, State University of New York, Brooklyn 11203.

A mixed culture recovery method indicates that enteric bacteria do not enter the viable but nonculturable state. Bogosian G, Morris PJ, O'Neil JP Appl Environ Microbiol 1998 May 64:5 1736-42

Abstract A new method, called the mixed culture recovery (MCR) method, has been developed to determine whether recovery of culturable bacterial cells from a population of largely nonculturable cells is due to resuscitation of the nonculturable cells from a viable but non-culturable state or simply to growth of residual culturable cells. The MCR method addresses this issue in that it involves the mixing of two easily distinguishable strains (e.g., lactose positive and negative) in such a way that large numbers of nonculturable cells of both strains are present together with a small number of culturable cells of only one strain, performing a nutrient addition resuscitation procedure, and then plating the cells to determine whether both cell types are recoverable. In repeated experiments with strains of Escherichia coli, Klebsiella pneumoniae, Enterococcus faecalis, Enterobacter aerogenes, and Salmonella choleraesuis, only cells of the culturable strain were recovered after application of various resuscitation techniques. These results suggest that the nonculturable cells were dead and that the apparent resuscitation was merely due to the growth of the remaining culturable cells.

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Recovery of culturability of an HOCl-stressed population of Escherichia coli after incubation in phosphate buffer: resuscitation or regrowth? Dukan S, Lévi Y, Touati D Appl Environ Microbiol 1997 Nov 63:11 4204-9

Abstract An Escherichia coli population harvested in exponential phase at about 10(8) cells/ml was treated in phosphate buffer with HOCl at concentrations ranging from 0.4 to 1 mg/liter (7.7 to 19 microM). The HOCl stress resulted in the appearance of three cell subpopulations: a majority of dead (nonrespiring) cells, a few culturable cells (10(2) to 10(4)), and about 10(7) viable but nonculturable cells. In the absence of any added exogenous

nutrient, a culturable population could be recovered after 1 day of incubation in phosphate buffer, and such a population would reach a cell density close to 10% of the initial density of the stressed population, whatever the initial number of survivors. When a small number of untreated cells were mixed with the stressed population, growth of the untreated cells was observed, demonstrating that damaged cells provided nutrients. Similarly, a filtrate and a disrupted-cell filtrate of the stressed population supported growth of untreated cells with the same efficiency. The number of CFU (untreated or stressed) at plateau phase depended on the initial density of the stressed cells. Taken together, these results suggest that recovery in phosphate buffer of an HOCl-stressed population is in large part due to growth of a few culturable cells at the expense of damaged cells. However, comparison of the growth rates of the stressed culturable population and of untreated bacteria growing in filtrate showed significantly faster growth of the stressed cells, a fact not fully compatible with the hypothesis that recovery is only the simple growth of survivors. We suggest, therefore, that in addition to growth of the few culturable stressed cells, there is repair and growth of some mildly injured viable but nonculturable cells.

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Fate of Enterobacter cloacae JP120 and Alcaligenes eutrophus AEO106(pRO101) in soil during water stress: effects on culturability and viability. Pedersen JC, Jacobsen CS Appl Environ Microbiol 1993 May 59:5 1560-4

Abstract A sandy loam soil near field capacity moisture content (psi = -0.050 MPa) or air dried (psi = -300 MPa) was inoculated with about 3 x 10(7) CFU of Enterobacter cloacae JP120 and Alcaligenes eutrophus AEO106(pRO101) per g and incubated in 40-g portions at 17 degrees C in closed or open Erlenmeyer flasks. In the field-moist soil, selective plating, direct viable counts, and DNA hybridization showed only minor changes in the numbers of E. cloacae and A. eutrophus cells with time (14 days), and the results obtained with the three detection methods generally agreed. In the air-dried soil, the majority of both bacteria were found as intact DNA-carrying cells that were neither culturable nor viable by the methods employed in this study. The numbers of culturable E. cloacae and A. eutrophus cells dropped to 10(5) and 10(2) CFU/g, respectively, 2 h after inoculation. Direct viable counts showed that only about 1% of the cells detected by immunofluorescence microscopy were viable, but a fraction of viable nonculturable cells of both bacteria was present. A. eutrophus did not tolerate desiccation as well as E. cloacae. Only a minor fraction of the two test organisms regained their culturability or viability after rewetting of the air-dried soil; the number of total heterotrophic culturable bacteria, however, increased more than 10-fold and reached 73% of the level found in the field-moist soil at day 14.

Department of Marine Ecology and Microbiology, National Environmental Research Institute, Roskilde, Denmark.

Resuscitation of viable but nonculturable Legionella pneumophila Philadelphia JR32 by Acanthamoeba castellanii. Steinert M, Emödy L, Amann R, Hacker J Appl Environ Microbiol 1997 May 63:5 2047-53

Abstract Legionella pneumophila is an aquatic bacterium and is responsible for Legionnaires' disease in humans. Free-living amoebae are parasitized by legionellae and provide the intracellular environment required for the replication of this bacterium. In low-nutrient environments, however, L. pneumophila is able to enter a non-replicative viable but nonculturable (VBNC) state. In this study, L. pneumophila Philadelphia I JR 32 was suspended in sterilized tap water at 10(4) cells/ml. The decreasing number of bacteria was monitored by CFU measurements, acridine orange direct count (AODC), and hybridization with 16S rRNA-targeted oligonucleotide probes. After 125 days of incubation in water, the cells were no longer culturable on routine plating media; however, they were still detectable by AODC and by in situ hybridization. The addition of Acanthamoeba castellanii to the dormant bacteria resulted in the resuscitation of L. pneumophila JR 32 to a culturable state. A comparison of plate-grown legionellae and reactivated cells showed that the capacity for intracellular

survival in human monocytes and intraperitoneally infected guinea pigs, which is considered a parameter for virulence, was not reduced in the reactivated cells. However, reactivation of dormant legionellae was not observed in the animal model.

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Detection of induced beta-galactosidase activity in individual non-culturable cells of pathogenic bacteria by quantitative cytological assay. Nwoguh CE, Harwood CR, Barer MR Mol Microbiol 1995 Aug 17:3 545-54

Abstract One Escherichia coli and two F' lac+ Salmonella strains were carbon and nitrogen stressed at 37 degrees C over 35 days in the presence or absence of chloramphenicol; the number, activity and culturability of cells in the resultant populations were studied. Active cells were enumerated by fluorescence microscopy after treatment with the lac inducer IPTG and cytological assay for beta-galactosidase. In all experiments, active and total cell counts remained within a three-fold range of each other and their initial values, while culturability fell by > 10(8)-fold and 10(3)-fold in chloramphenicol-treated and untreated preparations, respectively. Quantitative image analysis revealed different distributions of cell-specific fluorescence and indicated a progressive decline in the levels of induced enzyme activity in both E. coli and Salmonella enteritidis. It was concluded that the non-culturable cells studied retained inducible enzyme activity and that this activity did not result from a starvation-induced programme of gene expression. Whether or not such active but non-culturable cells are viable, they are clearly responsive and have the potential to influence their environment. The assay described can be applied to heterogeneous populations and environments and shows considerable potential for the study of gene expression at the single cell level.

Department of Microbiology, Medical School, Newcastle upon Tyne, UK.

Beta-D-galactosidase activity of viable, non-culturable coliform bacteria in marine waters. Davies CM, Apte SC, Peterson SM Lett Appl Microbiol 1995 Aug 21:2 99-102

Abstract The beta-D-galactosidase activity of viable but non-culturable (vnc) Escherichia coli cells in seawater was investigated using a rapid fluorimetric enzyme assay. Results from microcosm studies showed that loss of culturability did not necessarily result in loss of the ability to produce the galactosidase enzyme. Even when no culturable cells were detected, a positive enzyme assay response was observed and the activity of the inducible enzyme over time more closely reflected the number of vnc cells present.

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